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CALCIFICATION IN ECHINODERMS: EFFECTS OF TEMPERATURE AND DIAMOX ON INCORPORATION OF CALCIUM-45 *IN VITRO* BY REGENERATING SPINES OF *STRONGYLOCENTROTUS PURPURATUS* [1]

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Studies of the calcareous endoskeleton of echinoderms have been reviewed by Hyman (1955), Raup (1966), Swan (1966), Nicol (1967), and Nichols and Currey (1968). Recent work on the echinoderm endoskeleton has been carried out by numerous investigators including Ebert (1967, 1968), Towe (1967), Travis, Francois, Bonar, and Glimcher (1967), Donnay and Pawson (1969), Kobayashi and Taki (1969), Märkel and Titschack (1969), Nissen (1969), Pilkington (1969), Weber (1969), and Weber, Greer, Voight, White, and Roy (1969). To date, emphasis has been placed on various aspects of skeleton composition, morphology, crystallography, growth and regeneration. However, no quantitative studies have been reported on the physiology of skeleton formation in echinoderms (see Robertson, 1941; Nichols, 1964; and Nicol, 1967).

Due to the absence of quantitative studies on calcification in echinoderms, calcified tissues of the sea urchin, *Strongylocentrotus purpuratus* (Stimpson), were investigated with the objective of developing a method to study calcification rates under different conditions. Gradually, attention was focused on the spines. Preliminary experiments showed that intact spines incorporate variable and relatively low quantities of calcium-45. But, when spines were broken, rapid uptake of the label occurred as part of the process of skeleton regeneration. This observation suggested that regenerating spines might be useful as a calcifying system, and subsequent experiments confirmed this possibility.

Reported here are the results of experiments establishing regenerating spines of *S. purpuratus* as a useful tool to study calcification rates *in vitro* under different conditions with calcium-45 as a tracer. Using this calcifying system, the effects of temperature and a carbonic anhydrase inhibitor, Diamox (acetazolamide), on calcification rates are measured quantitatively.

Materials and Methods

Animals

Adult specimens of *S. purpuratus,* ranging in wet weight from 40 to 70 grams, were collected subtidally at Flat Rock Point, Los Angeles County, California,

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and maintained in the laboratory under constant room lighting in filtered, recirculating sea water at 14° C. Several species of brown algae were supplied as food.

Experimental procedures

Aboral, primary interambulacral spines of similar size were fractured with scissors 2 to 3 millimeters above the milled ring. As a result of this procedure, the stubs were protected from damage during regeneration by the surrounding forest of intact spines about 10 millimeters longer. Usually, 5 to 8 spines were fractured on each of the 10 plate rows of the interambulacra, which yielded from 50 to 80 stubs per urchin depending on animal size.

Fractured spines were incubated *in vivo* or *in vitro*. In the latter case, the stubs were removed from the urchin by severing the tissue attaching the base to the underlying tubercle. Incubations were carried out under constant room lighting at 15° C in covered, plastic dishes with a capacity of 500 or 1000 milliliters (Stoway utility dish, Southern California Plastic Co., Glendale). Calcium-45 with a specfic activity of 19.9 mc/mg was obtained from New England Nuclear, Boston, Massachusetts. In all experiments, aeration of the incubation medium was achieved by means of water-saturated air bubbled through an air stone. In some of the experiments, stubs were incubated *in vitro* simultaneously with stubs *in vivo,* but in separate compartments within the incubation chamber to prevent damage to them by the activities of the animal. The compartments were constructed from halves of plastic, histological coverslip boxes fastened to the inside of the chamber with methylene chloride just below the water level. To estimate ^{45}Ca incorporated into the skeleton by exchange, control spines previously bleached for several hours in 5.25% NaOCl (commercial strength Purex or Clorox) were fractured as above and added to the incubation medium in some of the experiments. Further details of each experiment are given in the Results section.

After incubation, stubs were sampled, bleached in Purex for 2 to 3 hours or longer to remove soft tissue and unincorporated ^{45}Ca, rinsed several times in distilled water adjusted to pH 7.0, and oven dried at 110° C on filter paper. Between rinsings, stubs were drained briefly on filtered paper. Care was taken to protect the delicate regenerated mineral from damage during handling. Dried stubs were mounted in modeling clay and stored until the activity of ^{45}Ca was determined.

Assay of ^{45}Ca activity

Individual stubs were fixed vertically with modeling clay on planchets. The tip of the regenerated portion of the stub was then positioned 1 mm below the center of a thin end-window Geiger-Müller tube (LND Corp., Oceanside, New York, #733 T) with the aid of an adjustable platform which permitted reproducible counting geometry. Activity was determined with a transistorized scaler (Nuclear Supplies Inc., Encino, California, Model SA-250). After incubation in ^{45}Ca for several days, some radioactivity was detectable on the periphery of the spine shaft in addition to that of the regenerated tip. Radioactivity on the shaft was reduced to background levels during counting by slipping a rubber disc with a small hole in the middle over the spine tip to a position just below the level of fracture,

care being taken not to touch the regenerated skeleton. The disc, of the same diameter as the planchet, was cut from the cuff of a rubber autopsy glove and pierced with a hot pin. Spines of different diameter were shielded by discs with holes of appropriate size. Each spine was counted for three minutes and the activity expressed as counts per minute per spine. In some of the experiments,

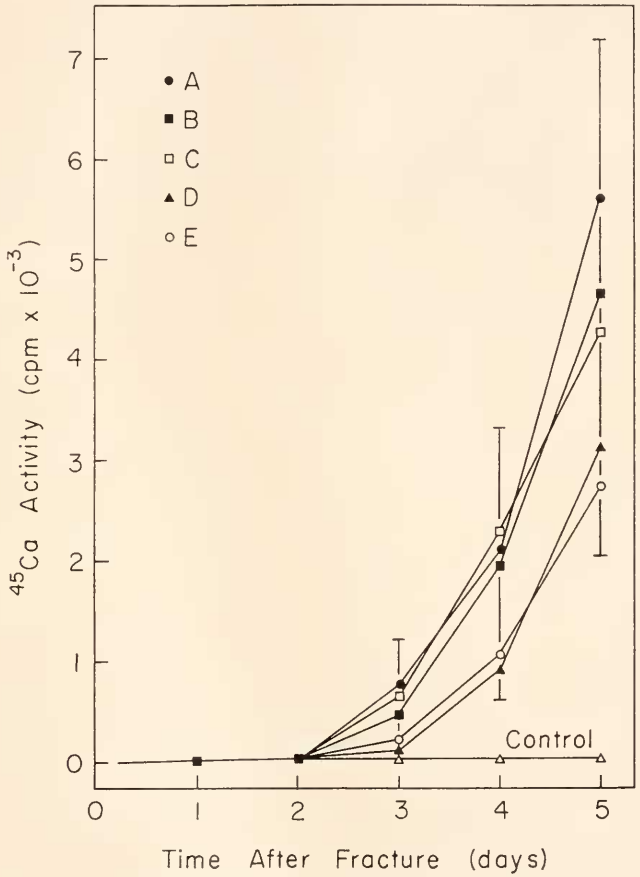

FIGURE 1. Kinetics of ^{45}Ca incorporation by regenerating spines of five specimens of *S. purpuratus* (A–E) incubated *in vivo* for five days following experimental fracture. Each point represents the mean value of 3 or 5 spines, with vertical bars indicating the range of all values. Control values are means of 3 bleached spines.

activity of ^{45}Ca in the incubation medium and coelomic fluid was determined in triplicate, 50 microliter aliquots which were dried on planchets and counted for three minutes. This activity was expressed as counts per minute per milliliter. All counts were corrected for background.

In experiments on the effects of temperature and Diamox on calcification rates, activity was determined independently by liquid scintillation techniques using the sample preparation method of Carr and Parsons (1962), adapted here to the assay

of spines labeled with ^{45}Ca. Scintillation counting eliminated error due to self absorption. Each stub was inverted over a liquid scintillation vial and the tip snipped off with scissors about 1 millimeter below the level of experimental fracture. This procedure insured complete recovery of all labeled mineral deposited on the fractured surface during regeneration. In some of the stubs employed in experiments on the effect of temperature on calcification rates, the second millimeter of the shaft was also removed and placed in a separate vial to estimate the activity of ^{45}Ca in the first millimeter which was included with the regenerated mineral as a consequence of the sampling procedure. Two milliliters of 0.5 *N* HCl were added to each vial to dissolve the calcareous fragments. The vials were then gradually heated to about 130° C to remove water and HCl. After cooling, 6 milliliters of a mixture of toluene and ethanol (5:1), and 5 g/L of 2,5-diphenyloxazole (PPO), were added to each vial. Activity of ^{45}Ca was determined at room temperature in a Beckman liquid scintillation counter, Model LS-233. Counts

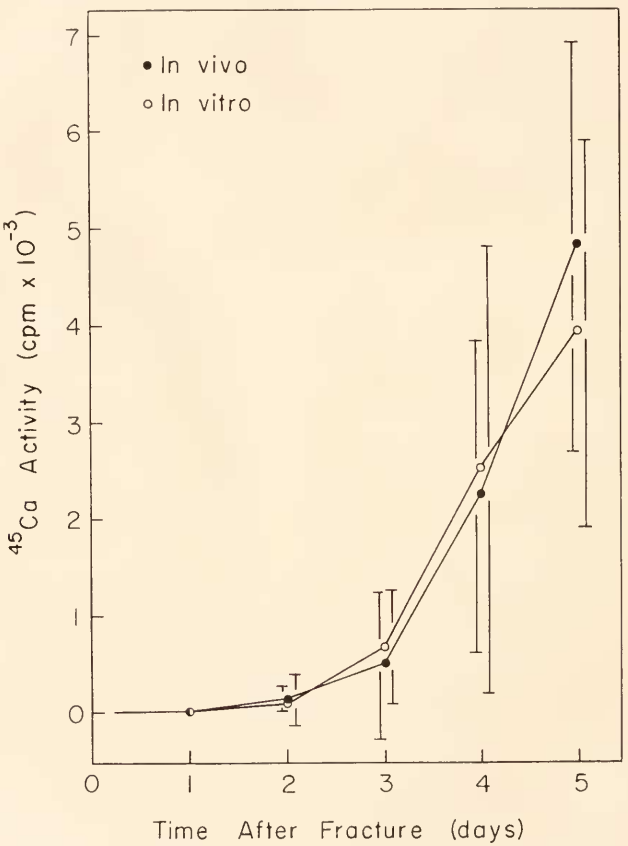

FIGURE 2. Kinetics of ^{45}Ca incorporation by regenerating spines of a single *S. purpuratus* incubated *in vivo* or *in vitro* for five days following experimental fracture. Each point represents the mean value of 5 spines, with vertical bars (left, *in vivo;* right, *in vitro*) indicating ± S.D.

were taken for five minutes, corrected for background, and expressed as counts per minute per spine. Error due to measurement of radioactivity was usually less than ± 5%.

Results

Comparison of calcification rates in vivo *and* in vitro

To determine when calcification was initiated during regeneration *in vivo,* five urchins with fractured spines were incubated individually in about 350 milliliters of sea water containing 1.0 μc/ml of ^{45}Ca. At daily intervals for five days, 3 or 5 stubs were removed from each animal and assayed for radioactivity.

Figure 1 shows that there was a lag period of about two days following fracture before ^{45}Ca was incorporated. During this time, the amount of label incorporated by regenerating spines was not significantly different from that incorporated in

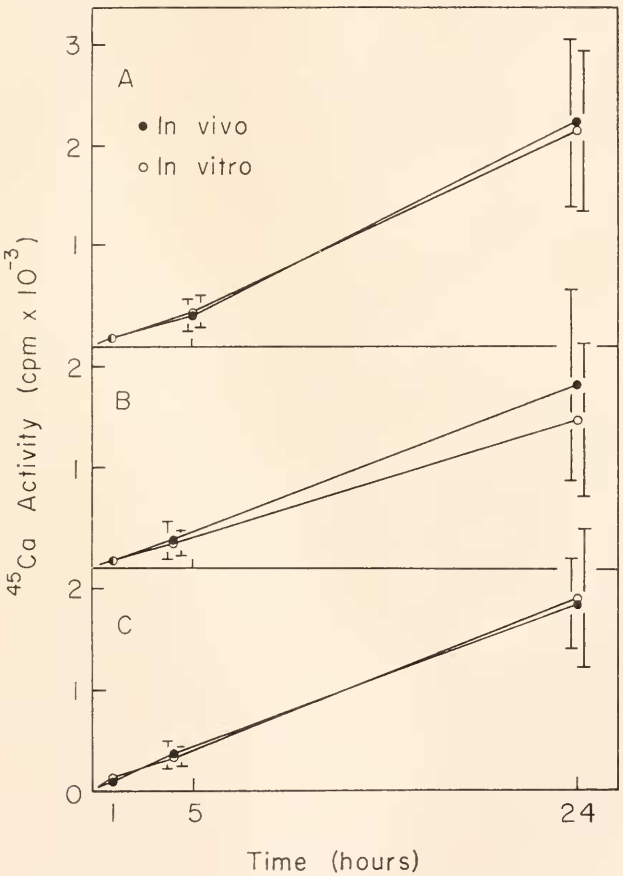

Figure 3. Kinetics of ^{45}Ca incorporation by spines of three specimens of *S. purpuratus* (A–C) incubated for 24 hours *in vivo* or *in vitro* after regeneration for four days *in vivo* in plain sea water following fracture. Each point represents the mean value of 10 spines, with vertical bars (left, *in vivo;* right, *in vitro*) indicating ± S.D.

bleached controls by exchange. Thereafter, uptake of ^{45}Ca by regenerating spines increased rapidly, with exchange accounting for only about 1% of the total activity incorporated four to five days after fracture.

During the first 24 hours' incubation *in vivo* there was a relatively rapid disappearance of ^{45}Ca activity from the incubation medium followed by a more gradual, nearly linear rate of removal. At the end of the incubation period, nearly 20% of the initial radioactivity was removed. In another experiment, the disappearance of radioactivity from the medium during the first 24 hours' incubation was concomitant with a rapid appearance of radioactivity in the coelomic fluid, which reached equilibrium with the medium after 30 to 36 hours' incubation.

To determine whether calcification of regenerating spines is initiated *in vitro*, fifty spines were fractured on a single urchin. Twenty-five stubs were then removed (explanted) and incubated *in vitro* simultaneously with the remainder *in vivo* in about 700 milliliters of sea water containing 1.0 μc/ml of ^{45}Ca. Five stubs were sampled daily from each group for five days and assayed for radioactivity.

Figure 2 shows that explanted stubs incorporated ^{45}Ca during regeneration *in vitro* in a manner similar to that of stubs *in vivo*. These explants also showed a two-day lag period during which relatively little ^{45}Ca was incorporated. Thereafter, a rapid increase in radioactivity was observed similar to that shown in Figure 1. A two-day lag period before rapid incorporation of ^{45}Ca was also observed in additional experiments carried out for up to five days with stubs incubated only *in vitro*.

To avoid the two-day lag period so that short term experiments could be conducted while calcification was in progress, spines were fractured on three urchins and allowed to regenerate *in vivo* for four days in plain sea water. One half of the number of stubs was then explanted from each urchin and incubated *in vitro* simultaneously with the remainder *in vivo* in 300 to 350 milliliters of sea water containing 1.0 μc/ml of ^{45}Ca. Ten stubs were sampled from both groups from each urchin at intervals up to 24 hours and assayed for radioactivity.

Figure 3 shows that ^{45}Ca incorporation by calcifying stubs over 24 hours was directly proportional to the length of incubation with no statistically significant difference between stubs incubated *in vivo* and those *in vitro*. Under conditions of the experiment, ^{45}Ca activity was detected in both groups after incubation for as little as one hour.

The results of these experiments indicate that, after regeneration for four days *in vivo* following fracture, explanted stubs could be used alone in further studies on calcification with ^{45}Ca as a tracer. The use of explants is advantageous since removal of ^{45}Ca from the medium due to uptake by the urchin is avoided. In addition, each urchin provides numerous regenerating stubs which can be presumed to be similar genetically and physiologically. Thus, it should be possible to conduct controlled experiments *in vitro* to evaluate quantitatively the effects of various parameters on calcification rates. In the present investigation, two parameters, (1) temperature, and (2) Diamox (acetazolamide), an inhibitor of the enzyme, carbonic anhydrase, were selected for study.

Effect of temperature on calcification rates in vitro

To determine the influence of temperature on calcification rates of regenerating spines *in vitro,* five dishes containing 200 milliliters of sea water and 1.5 μc/ml of ^{45}Ca were allowed to equilibrate for 24 hours at temperatures of 4.7°, 9.7°, 15°, 20°, and 26° C. Fifty stubs were explanted from each of four urchins after regeneration for four days *in vivo* in plain sea water following fracture. Ten stubs from each group of fifty were placed in each of the five dishes giving forty stubs per dish. The four groups of ten stubs in each dish were isolated from

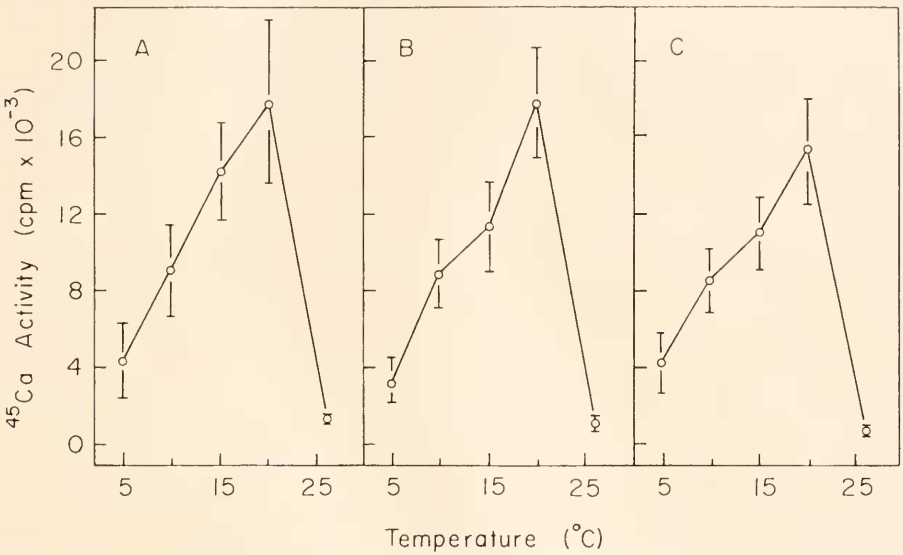

FIGURE 4. Incorporation of ^{45}Ca by spines of three specimens of *S. purpuratus* (A–C) incubated *in vitro* for 24 hours at various temperatures after regeneration for four days *in vivo* in plain sea water following fracture. Each point represents the mean value of 10 spines, with vertical bars indicating ± S.D. Data for a 4th urchin are similar to those shown, except that only a slight increase in ^{45}Ca incorporation occurred between 15° and 20° C.

one another by pieces of glass rod of $\frac{1}{8}$ inch diameter placed on the bottom of each dish. Temperatures varied by 1° C or less during the experiment. After incubation for 24 hours, all stubs from each animal were assayed for radioactivity by liquid scintillation.

Figure 4 shows that incorporation of ^{45}Ca by explants was directly proportional to temperature between 4.7° and 20° C, at which a maximum occurred. At 26° C, little incorporation of the label took place, and tissue on all stubs appeared to be partially decomposed at the end of the experiment. Mean values for the temperature coefficient (Q_{10}), and energy of activation (E) calculated over the intervals 4.7° to 9.7°, 9.7° to 15°, and 15° to 20° C, are 5.50, 2.08, 1.73; and 26,052, 11,491, and 8662 calories per mole, respectively, with overall means between 4.7° and 20° C, of 2.72, and 15,504 calories per mole, respectively, for the four urchins studied. Activity of ^{45}Ca measured in the second millimeter of the shaft of those stubs assayed was considered negligible.

Effect of Diamox on calcification rates in vitro

In living systems, carbonate ion ($CO_3^=$) can be obtained via bicarbonate ion (HCO_3^-), which is formed by the hydration or hydroxylation of CO_2 (see Wilbur, 1964), although it is not known with certainty which of these two mechanisms is operative at physiological pH (Maren, 1967). The enzyme, carbonic anhydrase, has been shown to catalyze the conversion of CO_2 to HCO_3^- (Meldrum and Roughton, 1933). If the rate of deposition of $CaCO_3$ is dependent upon the enzyme-catalyzed conversion of CO_2 to HCO_3^-, then the application of an appropriate inhibitor of carbonic anhydrase should depress the rate of calcification.

To test this hypothesis in calcifying sea urchin spines *in vitro,* a specific inhibitor of carbonic anhydrase, 2-acetylamino-1,3,4, thiadiazole-5-sulfonamide (Miller, Dessert, and Roblin, 1950), or acetazolamide, was obtained as the sodium salt, Diamox, from the Lederle Laboratories of the American Cyanamid Company,

Table I

The effect of various concentrations of a carbonic anhydrase inhibitor, Diamox (acetazolamide), on the incorporation of calcium-45 by spines of S. purpuratus *incubated* in vitro *for 24 hours after regeneration for four days* in vivo *in plain sea water following fracture. Shown are mean values in counts per minute ± S.D., for the number of spines in brackets. Probability values equal 0.01 or less (see text for statistical procedures).*

Animal	Control	Counts per minute			
		10^{-6} *M* Diamox	10^{-5} *M* Diamox	10^{-4} *M* Diamox	10^{-3} *M* Diamox
1	9596 ± 2404 (10)	3316 ± 968 (10)	4364 ± 2372 (10)	5080 ± 1604 (10)	4908 ± 2052 (10)
2	9000 ± 3102 (9)	3928 ± 1008 (10)	3544 ± 1392 (10)	5060 ± 1472 (10)	4280 ± 1656 (7)
3	9952 ± 1036 (10)	3764 ± 920 (10)	3784 ± 880 (10)	3968 ± 540 (10)	5112 ± 1344 (10)
Mean % inhibition ± S.E.		61 ± 5	58 ± 4	50 ± 9	53 ± 8

Pearl River, New York. A sea water solution of 4×10^{-3} *M* Diamox was adjusted to pH 8.0 and added to plain sea water in four plastic dishes giving final concentrations of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} *M*. A fifth dish without added inhibitor served as the control. The final volume in all dishes was 250 milliliters, with 1.0 μc/ml of ^{45}Ca. Fifty stubs were explanted from each of three urchins after regeneration for four days *in vivo* in plain sea water following fracture. Ten stubs from each group of fifty were placed in each of the five dishes giving thirty stubs per dish. The three groups of ten stubs in each dish were isolated from one another by pieces of glass rod as described earlier. Incubations were carried out at 15° C for 24 hours. All stubs from each animal were then assayed for radioactivity by liquid scintillation.

Table I shows that there was a statistically significant reduction in ^{45}Ca incorporation by explants treated with the inhibitor compared to the untreated controls. Probability values of 0.01 or less were obtained using the "t" test (Simpson, Roe, and Lewontin, 1960, page 176). The data show a fairly consistent effect of Diamox over a wide range of concentrations with a maximum mean inhibition of 61% at a concentration of 10^{-6} *M*.

Discussion

The results of kinetic studies reported here show that regenerating spines of the sea urchn, *S. purpuratus,* incorporate ^{45}Ca *in vitro* in a manner similar to that of spines from the same animal incubated simultaneously *in vivo.* The data demonstrate a lag period of about two days following experimental fracture before calcification is initiated. The lag period cannot be attributed to the time required for uptake of ^{45}Ca by the urchin, since a similar lag was also shown to occur during regeneration of fractured spines *in vitro.* The results of histological and histochemical studies of fractured spines during regeneration *in vivo* and *in vitro* indicate that the lag more likely reflects the time required for wound healing and reorganization of tissue at the site of fracture (Heatfield, unpublished). Following the lag period, a rapid incorporation of ^{45}Ca takes place *in vivo* and *in vitro.* The incorporation of ^{45}Ca following the lag period parallels the appearance and growth of new mineral in the form of "micro-spines" on the fractured surface of the spine shaft as observed with the light and scanning electron microscopes (Heatfield, 1969). In calcifying stubs which were prepared by allowing fractured spines to regenerate for four days *in vivo* in plain sea water, incorporation of ^{45}Ca *in vitro* was shown to be equivalent to that *in vivo* during incubation for 24 hours. The nearly linear rate of incorporation of the label during this period indicates that equilibrium between the tissues of regenerating spines and the incubation medium takes place rapidly (less than one hour at the spine tip).

Using explanted, fractured spines as a calcifying system after regeneration for four days *in vivo,* temperature was shown to have a marked effect on calcification rates *in vitro.* Incorporation of ^{45}Ca was directly proportional to temperature between 4.7° and 20° C, at which a maximum occurred. Little incorporation of the label took place at 26° C, which appears to be a lethal temperature. The temperature of the ocean in the vicinity of the collecting site ranges from about 13° to 20° C (Boolootian, 1961). This range is within that in which incorporation of ^{45}Ca was found to occur in the present work and indicates that temperature is not a limiting environmental factor in the regeneration process in spines of *S. purpuratus.* The temperature coefficient (Q_{10}) and the energy of activation (E) varied inversely with increasing temperature betwen 4.7° and 20° C. Valus of E obtained in the present study fall within the general range (5000 to 25,000 calories per mole, and higher) listed by Sizer (1943) for a large number of enzyme-catalyzed reactions, and by Crozier (1924) for a variety of physiological phenomena. In the present work, the variation of E (μ of Crozier) with temperature suggests that several rate-limiting reactions may be involved in the calcification process with the one in effect at any particular moment dependent upon the ambient temperature (see Crozier, 1924).

Few quantitative studies have been carried out on the effect of temperature on mineral deposition in other calcifying systems. Malone and Dodd (1967) found that incorporation of ^{45}Ca into whole shells of the bivalve mollusc, *Mytilus edulis,* varied directly with temperature over the range, 5° to 23.5° C. Incorporation due to exchange was not estimated and it was concluded that activity of ^{45}Ca in shells incubated at the lower temperatures might not be due to skeletogenesis. In the present study, incorporation of ^{45}Ca at 4.7° C was about $\frac{1}{4}$ of the

maximum observed at 20° C, indicating that significant deposition of mineral takes place in regenerating spines of *S. purpuratus* even at relatively low temperatures. Porcella, Rixford, and Slater (1969) found that uptake of ^{45}Ca by the fresh water crustacean, *Daphnia magna*, was temperature dependent, and obtained mean values for E of 15,000 calories per mole between temperatures of 10° and 25° C. In the study reported here, a similar mean value of E of 15,504 calories per mole was obtained between 4.7° and 20° C. In the reef coral, *Pocillopora damicornis*, Clausen (unpublished, cited in Lenhoff, Muscatine, and Davis, 1968) measured the effect of temperature on the incorporation of ^{45}Ca, and found an exponential increase in radioactivity between 12° and 25° C, with a Q_{10} of 12.7 or 6.7 depending on the length of incubation. Incorporation at 12° was about 1/13 that at 25° C. Above 25° C, a decline in calcification rate was observed. Values for E of 43,000 and 33,000 calories per mole were obtained by Clausen depending on the length of incubation. These values are about twice as high as those obtained in the present study and by Porcella *et al.* (1969), and indicate that the rate of calcification in this species of coral is very sensitive to changes in temperature.

The enzyme, carbonic anhydrase, is generally believed to be important in the biological deposition of calcium carbonate as inferred from the results of experiments on the effect of carbonic anhydrase inhibitors on the rate of mineral deposition. The data reported here show a statistically significant reduction in the incorporation of ^{45}Ca by regenerating spines of *S. purpuratus* incubated *in vitro* in the presence of a carbonic anhydrase inhibitor, Diamox (acetazolamide). Inhibition of 50% to 61% was obtained over a concentration range of 10^{-3} to 10^{-6} *M*. From these results it is inferred that carbonic anhydrase plays a role in calcification of regenerating spines of *S. purpuratus* though no attempt has yet been made to determine the presence of this enzyme in spine tissues. Inhibition of calcification has been observed in other organisms in which carbonic anhydrase activity was detected. Wilbur and Jodrey (1955) obtained up to 48% inhibition of ^{45}Ca incorporation by the oyster, *Crassostrea virginica*, in the presence of 4×10^{-5} *M* Diamox. Costlow (1959) observed an inhibition of shell growth in the barnacle, *Balanus improvisus*, after treatment with Diamox at concentrations ranging from 3.7×10^{-4} to 2.99×10^{-5} *M*. In four species of reef coral, Goreau (1959) found an inhibition of ^{45}Ca incorporation of 51% to 80% in experiments conducted in the light with Diamox at a concentration of 10^{-3} *M*. Isenberg, Lavine, and Weissfellner (1963) obtained essentially complete inhibition of mineralization in the coccolithophorid, *Hymenomonas* sp. after exposure to 10^{-3} *M* Diamox, though the demonstration of carbonic anhydrase was not attempted in this organism. After injection of Diamox into the hen, *Gallus domesticus*, Bernstein, Nevalainen, Schraer, and Schraer (1968) measured a reduction in egg-shell weight of approximately 59%.

The results of these studies with Diamox, with the possible exception of Costlow (1959) and Isenberg *et al.* (1963), indicate that carbonic anhydrase is generally not indispensable to skeletogenesis, since deposition of mineral occurs, though at a reduced rate. Concentrations of Diamox which have a marked effect on calcification rates do not appear to be toxic (see Wilbur and Jodrey, 1955; Costlow, 1959; Goreau, 1959; Freeman, 1960; Isenberg *et al.* (1963); and Maren, 1967). In the presence of another inhibitor, 2-benzothiazolesulfonamide,

Stolkowski (1948) observed an inhibition of growth in the larval skeleton of the sea urchins, *Paracentrotus lividus* and *Arbacia aequituberculata.* However, complete inhibition of mineral deposition was obtained with this drug in *C. virginica* by Wilbur and Jodrey (1955), and in the fresh water snail, *Physa heterostropha,* by Freeman (1960), which led these workers to conclude that the action of 2-benzothiazolesulfonamide was not limited to carbonic anhydrase.

In calcifying systems where deposition of mineral proceeds at a slow rate and the enzyme-catalyzed hydration or hydroxylation of CO_2 is not rate-limiting, then the application of inhibitors of carbonic anhydrase may not show an effect. Such a result was obtained by Freeman (1960) in studies of shell growth of *P. heterostropha* grown at different rates in the presence of Diamox.

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Summary

1. Calcification during regeneration of experimentally fractured spines of the sea urchin, *Strongylocentrotus purpuratus* (Stimpson), was studied quantitatively under different conditions with calcium-45 as a tracer.

2. Fractured spines rapidly incorporated ^{45}Ca *in vivo* or *in vitro* after a lag period of about two days. The lag period is attributed to wound healing and reorganization of tissue at the site of fracture.

3. Additional experiments were conducted while calcification was in progress by allowing fractured spines to regenerate for four days *in vivo* followed by incubation in ^{45}Ca *in vivo* or *in vitro* up to 24 hours. In these experiments incorporation of the label was nearly linear with time and no significant difference was observed in the rate of uptake of ^{45}Ca between regenerating spines incubated *in vivo* and those from the same urchin incubated simultaneously *in vitro.*

4. Incorporation of ^{45}Ca *in vitro* was directly proportional to temperature between 4.7° and 20° C, at which a maximum occurred. A temperature of 26° C appeared to be lethal and little incorporation of ^{45}Ca took place. Values of Q_{10} and the energy of activation varied inversely with temperature, with overall means of 2.72 and 15,504 calories per mole, respectively, between 4.7° and 20° C.

5. Diamox (acetazolamide) at concentrations from 10^{-3} to 10^{-6} *M,* inhibited incorporation of ^{45}Ca *in vitro* by 50% to 61%. It is inferred from these results that carbonic anhydrase is involved in calcification of regenerating spines of *S. purpuratus.*

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